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Microfiche No.

OTS0528817

New Doc I.D.

86-910000641

Old Doc I.D.

Date Produced

6/01/78

Date Recieved

1/24/91

TSCA section

8D

Submitting Organization

W.R. GRACE & COMPANY

Contractor

LITTON BIONETICS INC

Document Title

MUTAGENICITY EVALUATION OF L-2001 IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY (FINAL REPORT) WITH COVER LETTER DATED 011691

Chemical Category

TOLUENE DIISOCYANATE (26471-62-5)

CONTAINS NO CBI

GRACE 86-910000641

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91 JAN 25 AM 9:44

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Attn: Health and Safety Reporting Rule (Notification/Reporting)

Please find attached 8(d) health and safety reports for mixtures processed containing toluene diisocyanate (CAS #26471-62-5), 4,4-Diphenylmethane diisocyanate (CAS #101-68-8) and 1,6-Diisocyanatohexane (CAS #822-06-0). Grace is submitting these reports for late filing since their submittal may have been subject to the isocyanates 10-year call-in of June 1, 1987.

We have reason to believe that some of these reports may have previously been submitted to EPA as attachments to PMN submissions. However, Grace is filing them as a precautionary measure to insure EPA's receipt.

These reports are being submitted for:

W. R. Grace & Co.-Conn.
Washington Research Center
7379 Route 32
Columbia, MD 21044

Sincerely,

J. W. Raksis

A:\JR91-013/lw

Attachments - 20

86910000638

Liquid

CONTAINS NO CBI

Toluene Diisocyanate
26471-62-5
1,6 Diisocyanatohexane
822-06-0

MUTAGENICITY EVALUATION OF

L-2001

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

FINAL REPORT

SUBMITTED TO:

W.R. GRACE AND COMPANY
7379 ROUTE 32
COLUMBIA, MD 21044

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20839

JUNE 1978

PREFACE

This report contains a summary of the data compiled during the evaluation of the test material. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IV and provides sponsor and compound identification information, type of assay and the protocol reference number. All protocol references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V identifies the tables and/or figures containing the data used by the Study Director in interpreting the test results (item VI).

The second part of the report, entitled PROTOCOL, describes, in detail, the materials and procedures employed in conducting the evalution. This part of the report also contains evaluation criteria, standard operating procedures and any appendices. These are included to acquaint the sponsor with the methods used to develop and analyze the test results. All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology. Copies of raw data will be supplied to the sponsor upon request.

I. SPONSOR: W.R. Grace & Company

II. MATERIAL*

A. Identification: L-2001

B. Date Received: October 10, 1977

C. Physical Description: Viscous yellow liquid

III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay

IV. PROTOCOL NO.: OMT-106

V. RESULTS

The data presented in Table 1 show the concentrations of the test compound employed, number of mutant clones obtained, surviving populations after the expression period, and calculated mutation frequencies. All calculations are performed by computer program.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test material, L-2001, was prepared as a stock solution in DMSO (32 µl/ml) and diluted in DMSO to obtain a series of concentrations employed for toxicity determination. A concentration of 0.32 µl/ml was clearly cytotoxic for the L5178Y cells. The maximum tolerated concentration was between 0.12 µl/ml and 0.16 µl/ml. Two separate tests were conducted and the concentration range employed was from 0.0056 µl/ml to 0.16 µl/ml.

The results of the first tests are shown in Table 1. The data appear slightly elevated across several doses but do not show a dose related trend. Under nonactivation test conditions, a single data point (0.04 µl/ml) was 2.5 times the solvent control, but not the negative control. The same was true for the activation test results. The data were considered suggestive enough for a repeat test.

The results of the second test are shown in Table 2. The data from this test were also seen to fluctuate above the control data in the activation test. Again, no consistent trend in the data was observed and the results were considered to be random variation since adequate toxicity was obtained in this run.

*Information was supplied by the sponsor. If information was not indicated by the sponsor, N.I. was entered.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

It was concluded that L-2001 was not mutagenic for the L5178Y (TK +/-) cells under the conditions of this evaluation. Increased mutation frequencies were observed in two runs of the compound but the data appeared to be random variation and not consistent with compound-induced mutagenesis.

Submitted by:

Study Director

5/31/78

Dale W. Matheson, Ph.D.
Associate Director and
Section Chief
Mammalian Genetics
Department of Genetics
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Date

Reviewed by:

6/1/78

David Brusick, Ph.D.
Director
Department of Genetics
and Cell Biology

Date

SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: L-2001
B. SOLVENT: DMSO
C. TEST DATE: 02/27/78
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) OR NANOLITERS(NL) PER MILLILITER.

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY** X 10E-6
NONACTIVATION											
SOLVENT CONTROL	---	---	15.8	12.5	13.9	100.0	32.5	143.5	100.0	100.0	22.0
NEGATIVE CONTROL	---	---	20.6	12.4	10.4	96.8	49.0	158.0	110.1	106.5	31.0
EMS 0.5UL/ML	---	---	8.4	14.8	9.4	42.6	520.0	62.0	43.2	18.4	838.7
TEST COMPOUND											
0.010000000 UL/ML	---	---	15.6	13.0	14.8	109.3	37.0	118.0	82.2	89.9	31.4
0.020000000 UL/ML	---	---	20.6	9.2	14.4	99.4	46.0	175.0	122.0	121.2	26.3
0.040000000 UL/ML	---	---	12.6	15.0	10.4	71.6	80.0	142.0	99.0	70.9	56.3
0.080000000 UL/ML	---	---	9.4	5.0	3.0	5.1	80.0	161.0	112.2	5.8	49.7
0.160000000 UL/ML	---	---	0.6	0.8	5.8	2.7	30.0	149.0	103.8	2.8	20.1
ACTIVATION											
SOLVENT CONTROL	MOUSE	LIVER	16.1	14.2	11.2	100.0	27.0	115.0	100.0	100.0	23.0
NEGATIVE CONTROL	MOUSE	LIVER	18.0	10.8	12.2	92.6	59.0	115.0	100.0	92.6	51.3
DMN 0.5UL/ML	MOUSE	LIVER	7.6	4.6	6.4	8.7	270.0	18.0	15.7	1.4	1500.0
TEST COMPOUND											
0.010000000 UL/ML	MOUSE	LIVER	17.4	10.0	14.4	97.9	C	C			C
0.020000000 UL/ML	MOUSE	LIVER	11.0	16.4	12.6	88.8	34.0	146.0	127.0	112.7	23.3
0.040000000 UL/ML	MOUSE	LIVER	11.6	16.2	13.2	96.9	67.0	75.0	65.2	63.2	89.3
0.080000000 UL/ML	MOUSE	LIVER	8.4	10.2	13.2	44.2	82.0	92.0	80.0	35.3	89.1
0.160000000 UL/ML	MOUSE	LIVER	0.8	2.4	21.0	7.1	32.0	102.0	88.7	6.3	31.4

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** (MUTANT CLONES / VIABLE CLONES) X 10E-6
C = Contamination

TABLE 2

4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: L-2001
B. SOLVENT: DMSO
C. TEST DATE: 04/16/78
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) OR NANOLITERS(NL) PER MILLILITER.

TEST		S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY** X 10E-6
NONACTIVATION												
SOLVENT CONTROL		---	---	10.5	10.3	8.6	100.0	82.5	369.0	100.0	100.0	22.0
NEGATIVE CONTROL		---	---	6.2	20.6	9.8	134.6	85.0	271.0	73.4	98.8	31.4
EMS .5UL/ML		---	---	4.6	12.8	9.6	60.8	768.0	139.0	37.7	22.9	552.5
TEST COMPOUND												
0.00560	UL/ML	---	---	9.6	10.8	8.8	98.1	74.0	338.0	91.6	89.9	21.9
0.01500	UL/ML	---	---	7.6	9.8	8.8	70.5	85.0	463.0	125.5	88.4	18.4
0.04000	UL/ML	---	---	3.0	5.2	13.8	23.1	101.0	284.0	77.0	17.8	35.6
ACTIVATION												
SOLVENT CONTROL		RAT	LIVER	4.6	11.8	9.3	100.0	121.0	330.0	100.0	100.0	36.0
NEGATIVE CONTROL		RAT	LIVER	4.4	8.8	10.8	82.8	133.0	338.0	102.4	84.8	39.3
DMN .5UL/ML		RAT	LIVER	4.4	1.8	2.4	6.4	159.0	15.0	4.5	0.3	1060.0
TEST COMPOUND												
0.00560	UL/ML	RAT	LIVER	2.8	10.0	17.4	104.2	158.0	200.0	60.6	63.2	79.5
0.01500	UL/ML	RAT	LIVER	2.0	12.6	14.0	104.8	139.0	276.0	83.6	87.7	50.4
0.04000	UL/ML	RAT	LIVER	3.6	7.8	16.8	78.8	147.0	221.0	67.0	52.8	66.5
0.08000	UL/ML	RAT	LIVER	1.4	5.0	15.4	44.4	85.0	276.0	83.6	37.1	30.8
0.12000	UL/ML	RAT	LIVER	0.8	0.4	9.0	15.4	180.0	258.0	78.2	12.0	69.8

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** (MUTANT CLONES / VIABLE CLONES) X 10E-4

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for specific locus forward mutation induction in the L5178Y thymidine kinase (TK) mouse lymphoma cell assay.

2. MATERIALS

A. Indicator Cells

The cells used in this study were derived from Fischer mouse lymphoma cell line L1578Y. The cells are heterozygous for a specific autosomal mutation at the TK locus and are bromodeoxyuridine BrdU sensitive. Scoring for mutation was based on selecting cells that have undergone forward mutation from a TK+/- to a TK-/- genotype by cloning them in soft agar with BrdU.

B. Media

The cells were maintained in Fischer's medium for leukemic cells of mice with 10% horse serum and sodium pyruvate. Cloning medium consisted of Fischer's medium with 20% horse serum, sodium pyruvate, and 0.3% Noble agar. Selection medium was made from cloning medium by the addition of 7.5 mg BrdU to 100 ml cloning medium.

C. Control Compounds

1. Negative Control

The solvent in which the test compound was dissolved was used as a negative control and is designated as solvent control in the data table. The actual solvent is listed in Table 1 of Section V Results.

2. Positive Control

Ethylmethanesulfonate (EMS), which induces mutation by base-pair substitution, was dissolved in culture medium and used as a positive control for the nonactivation studies at a final concentration of 0.5 µl/ml.

Dimethylnitrosamine (DMN), which induces mutation by base-pair substitution and requires metabolic biotransformation by microsomal enzymes, was used as a positive control substance for the activation studies at a final concentration of 0.5 µl/ml.

3. EXPERIMENTAL DESIGN

A. Toxicity

The solubility, toxicity, and doses for all chemicals were determined prior to screening. The effect of each chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete growth medium. Toxicity was measured as loss in growth potential of cells induced by a 4-hr exposure to the chemical followed by a 24-hr expression period in growth medium. A minimum of 4 concentrations was selected from the concentration range by using as the highest dose a level that showed a reduction in growth potential. At least 3 lower doses, including levels which were below the toxic range, were added. Those compounds that were relatively nontoxic to cells growing in suspension were tested at concentrations of up to 10 mg/ml when solubility permitted. Toxicity produced by chemical treatment was monitored during the experiment.

B. Assays

1. Nonactivation Assay

The procedure used is a modification of that reported by Clive and Spector (1975). Prior to each treatment, cells were cleansed of spontaneous TK-/- by growing them in a medium containing thymidine, hypoxanthine, methotrexate, and glycine (THMG). This medium permits the survival of only those cells that produce TK, and can therefore utilize the exogenous thymidine from the medium. The test compound was added to the cleansed cells in growth medium at the predetermined doses for 4 hr at 37 C on a rocker. The treated cells were washed, fed, and allowed to express in growth medium for 3 days. At the end of this expression period. TK-/- mutants were detected by cloning the cells in the selection medium for 10 days. Surviving cell populations were determined by plating diluted aliquots in nonselective growth medium.

2. Activation Assay

The activation assay differs from the nonactivation assay in the following manner only. S9 was added to 10 milliliters growth medium containing appropriate cofactors and the desired number of cleansed cells. After adding the test compound, the flask was incubated with agitation for 4 hr at 37 C. The incubation period was terminated by washing the cells twice with growth medium. The washed treated cells were then allowed to express for 3 days and were cloned as indicated for the nonactivation cells.

3. EXPERIMENTAL DESIGN (Continued)

C. Preparation of 9,000 x g Supernatant

Male, random bred mice (HA/ICR) were killed by cranial blow, decapitated, and bled. The liver was immediately dissected from the animal using aseptic technique and placed in ice-cold 0.25 M sucrose buffered with Tris buffer at pH 7.4. When an adequate number of livers had been collected, they were washed twice with fresh buffered sucrose and completely homogenized. The homogenate was centrifuged for 20 min at 9,000 x g in a refrigerated centrifuge. The supernatant from this centrifuged sample was retained and frozen at -80°C until used in the activation system. This microsome preparation was added to a core reaction mixture to form the activation system described below:

Component	Final Concentration/ml
TPN (sodium salt)	6 µmol
Isocitric acid	35 µmol
Tris buffer, pH 7.4	28 µmol
$MgCl_2$	2 µmol
Homogenate S9 fraction	100 µl

D. Screening

A mutation index was derived by dividing the number of clones formed in the BrdU-containing selection medium by the number found in the same medium without BrdU. The ratio was then compared to that obtained from other dose levels and from positive and negative controls. Colonies were counted on an electronic colony counter that resolves all colonies greater than 200 microns in diameter.

4. EVALUATION CRITERIA

Several criteria have been established which, if met, provide a basis for declaring a material genetically active in the Mouse Lymphoma Forward Mutation Assay. These criteria are derived from a historical data base and are helpful in maintaining uniformity in evaluations from material to material, and run to run. While these criteria are reasonably objective, a certain amount of flexicility may be required in making the final evaluation since absolute criteria may not be applicable to all biological data.

4. EVALUATION CRITERIA (Continued)

A compound is considered mutagenic in this assay if:

- A dose-response relationship is observed over 3 of the 4 dose levels employed.
- The minimum increase at the high level of the dose-response curve is at least 2.5 times greater than the solvent and/or negative control values.
- The solvent and negative control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based on consideration of the concurrent solvent and negative control values run with the experiment in question. Positive control values are not used as reference points, but are included to ensure that the current cell population responds to direct and promutagens under the appropriate treatment conditions.

Occasionally, a single point within a concentration range will show an increase 2.5 times greater than the spontaneous background. If the increase is at the high dose, is reproducible, and if an additional higher dose level is not feasible because of toxicity, the chemical can be considered mutagenic. If the increase is internal within the dose range and is not reproducible, the increase will normally be considered aberrant. If the internal increase is reproducible, several doses clustered around the positive concentration will be examined to either confirm or reject the reliability of the effect.

As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.

REFERENCES

Clive, D. and Spector, J.F.S. (1975). Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31, 17-29.

5. STANDARD OPERATING PROCEDURE

All data will be entered in ink (no pencil).

All changes or corrections in entries will be made with a single line through the change, and an explanation for the change must be written.

All calculations (weights, dilutions, dose calculations, etc.) will be shown on data records.

All data entries will be dated and initialed.

All laboratory operations will be written out in standard protocol manuals. These manuals will be present in each laboratory area.

Deviations from any established protocol will be described and justified.

Data will be stored in bound form (notebooks or binders). These bound data books will be reviewed by the appropriate Section Heads.

Chemicals submitted for testing will have date of receipt and initials of entering person.

Lot numbers for all reference mutagens, solvent, or other materials used in assays will be recorded.

Animal orders, receipts, and identification will be recorded and maintained such that each animal can be traced to the supplier and shipment. All animals on study will be properly identified.

A copy of the final report plus all raw data and support documents will be permanently stored in the archival system of Litton Bionetics, Inc.

Current curricula vitae and job descriptions will be maintained on all personnel involved in the study.

CERTIFICATE OF AUTHENTICITY

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